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Comparison of anti-*Campylobacter* activity of free thymol and thymol-β-D-glucopyranoside in absence or presence of β-glycoside-hydrolysing gut bacteria

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ABSTRACT

Thymol is a natural product that exhibits antimicrobial activity *in vitro* but *in vivo* results indicate that absorption within the proximal alimentary tract precludes its delivery to the distal gut. Presently, the anti-*Campylobacter* activity of thymol was compared against that of thymol-β-D-glucopyranoside, the latter being resistant to absorption. When treated with 1 mM thymol, *Campylobacter coli* and *jejuni* were reduced during pure or co-culture with a β-glycoside-hydrolysing *Parabacteroides distasonis*. Thymol-β-D-glucopyranoside treatment (1 mM) did not reduce *C. coli* and *jejuni* during pure culture but did during co-culture with *P. distasonis* or during mixed culture with porcine or bovine faecal microbes possessing β-glycoside-hydrolysing activity. Fermentation acid production was reduced by thymol-β-D-glucopyranoside treatment, indicating that fermentation was inhibited, which may limit its application to just before harvest. Results suggest that thymol-β-D-glucopyranoside or similar β-glycosides may be able to escape absorption within the proximal gut and become activated by bacterial β-glycosidases in the distal gut.
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1. Introduction

Campylobacter spp. are a leading cause of bacterial derived foodborne illness worldwide (Epps et al., 2013). According to the United States Centers for Disease Control and Prevention, consumption of improperly cooked meats or unpasteurized dairy products contaminated with *Campylobacter* is a major cause of human infections (CDC, 2013). *Campylobacter* can colonize the digestive tracts of food-producing animals at high prevalence; consequently, the potential for contamination of carcasses during processing is recognised as a major concern of the food animal industry (Epps et al., 2013; Horrocks, Anderson, Nisbet, & Ricke, 2009). At present, however, few strategies are available to prevent contamination of carcasses at the processing plant and fewer yet are available to prevent infection and carriage of *Campylobacter* in animals on the farm (Horrocks et al., 2009). Consequently, the food animal industry is continually looking for new strategies capable of reducing the carriage of these pathogens on the farm and during processing.

Thymol (2-isopropyl-5-methylphenol) is a natural essential oil that markedly inhibits the survivability of *Campylobacter* in pure and mixed culture *in vitro* (Anderson et al., 2009). However, *in vivo* studies have demonstrated that thymol is extensively absorbed or degraded within the stomach and small intestine, thereby precluding delivery of this compound to the caecum and large intestine where *Campylobacter* primarily reside (Anderson et al., 2012; Michiels et al., 2008, 2010). It is reasonable to hypothesize, however, that thymol conjugates bound to glucose via a β-glycosidic bond may make the bound form of thymol more

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resistant to degradation and absorption within the proximal alimentary tract of monogastrics, where β-glycosidase activity would be minimal. In support of this hypothesis, Petrujkić and colleagues demonstrated that thymol-β-D-glucopyranoside is absorbed much less effectively than is free thymol in everted porcine stomach or small intestinal segments which suggests that appreciable quantities of the thymol-conjugate should pass to the lower gastrointestinal tract (Petrujkić et al., 2013). Upon arrival at the lower gastrointestinal tract, however, it is expected that microbial expressed β-glycosidase activity would be sufficient to liberate thymol from the glycoside, thereby allowing it to be active. Accordingly, the objective of this research project was to compare the bactericidal activities of free thymol and the conjugated form, thymol-β-D-glucopyranoside, on *Campylobacter jejuni* and *Campylobacter coli* during pure culture and during co-culture with a β-glycoside-hydrolysing gut bacterium, *Parabacteroides distasonis*, as well as during culture with mixed populations of swine and bovine gut bacteria.

2. Materials and methods

2.1. Microbe sources

C. coli was isolated from a dairy cow (Callaway et al., 2005) and *C. jejuni* was isolated from a broiler processing facility (Byrd et al., 2001). The *Campylobacter* strains were stored during long term maintenance in CryoCare™ Bacterial Preservers (Key Scientific Products, Round Rock, TX, USA) according to the manufacturer's instructions. Each strain was resuscitated for each experiment via initial 24 h culture (39 °C) in nonantibiotic-supplemented Bolton broth (Oxoid Ltd, Basingstoke, Hampshire, UK) and subsequent plating onto Campy Cefex agar prepared and used as described by Stern, Wojton, and Kwiater (1992). Isolated colonies, propagated after 48 h incubation at 42 °C, were picked and inoculated into Bolton broth supplemented with 0.33 μg of cefoperazone and 200 μg of cycloheximide per ml which, after 24 h of incubation (39 °C), served as the stock culture for experiments described below. *P. distasonis*, formerly classified as *Bacteroides distasonis* (Sakamoto & Benno, 2006), was obtained from porcine caecal contents grown in a continuous flow culture (Harvey et al., 2002). Throughout our studies, incubations in Bolton broth were performed at 39 °C to approximate the normal internal body temperature of cattle and swine.

2.2. Inhibition of Campylobacter during pure or co-culture with β-glycoside-hydrolysing P. distasonis

The effects of thymol (Sigma–Aldrich, St. Louis, MO, USA) and thymol-β-D-glucopyranoside (Christof Senn Laboratories, Dielsdorf, Switzerland) on *C. coli* and *C. jejuni* during both pure culture and co-culture with *P. distasonis* were tested in two experiments conducted on separate days. Cultures in antibiotic-free Bolton broth were prepared anaerobically by boiling and then cooling on ice under a continuous stream of 100% N_2 gas. Once cooled, 5% (v/v) laked horse blood (Lampire Biological Laboratories Pipersville, PA, USA) was added and 10 ml volumes of the medium were aseptically distributed to pre-sterilized 18 × 150 mm crimp top tubes, using a modification of the Hungate anaerobic method, as described by Bryant (1972). Triplicate sets of tubes were then supplemented with small volumes (0.1 and 0.16 ml in the first and second experiments, respectively) of stock solutions of thymol or thymol-β-D-glucopyranoside (prepared in 10% ethanol), to achieve 1 mM, which was determined to be an effective inhibitory concentration of free thymol in an earlier study (Anderson et al., 2009). In each experiment, triplicate sets of tubes were also supplemented with the same volumes of 10% ethanol for use as controls. The tubes were inoculated (0.2% vol/vol) with *C. coli* in the first experiment or *C. jejuni* in the second experiment and the respective co-cultures were also inoculated with *P. distasonis* (0.2% vol/vol). The co-culture tubes were closed and incubated upright without agitation at 39 °C. Fluids, collected at 0, 6, 24 and 48 h for pure and co-cultures with *C. coli* and at 0, 24 and 48 h with pure and co-cultures with *C. jejuni*, were analysed colorimetrically for determination of ammonia (Chaney & Marbach, 1962) and bacteriologically for viable cell count enumeration of *Campylobacter*, as routinely done in this laboratory (Gutierrez-Bañuelos et al., 2011; Krueger et al., 2008). Briefly, samples were serially diluted (10-fold) in 0.1 M sodium phosphate buffer (pH 7.34) and spread onto Campy Cefex agar. Inoculated Campy Cefex agar plates were incubated at 42 °C under a microaerophilic gas phase. Colonies were counted after 48 h of incubation at 42 °C. Gas chromatographic (GC) analyses, for free thymol (Petrujkić et al., 2013) and pH measurements, were done on remaining volumes of culture fluids from the pure and co-cultures of *C. jejuni*, using a pH meter.

2.3. Inhibition of Campylobacter during culture with mixed populations of porcine or bovine faecal microbes

To test the effect of thymol and thymol-β-D-glucopyranoside within mixed populations of gut bacteria, *C. coli* was cultured at 39 °C under N_2 in anaerobic Bolton broth inoculated with freshly voided porcine faeces obtained from a mature sow. Similarly, in a separate experiment *C. jejuni* was cultured at 39 °C under N_2 in anaerobic Bolton broth inoculated with freshly voided bovine faeces obtained from a mature cow. Faecal specimens were inoculated into their Bolton broth at 0.2 g per 100 ml and *C. coli* or *C. jejuni* were inoculated into the respective suspensions at 0.2% vol/vol. In these mixed culture experiments, test compounds were added in small volumes (0.10 ml) of concentrated stock solutions of thymol or thymol-β-D-glucopyranoside (each dissolved in a 62% ethanol solution) to achieve 1.0 mM final concentration. Equal volumes of water or 80% ethanol were similarly added to control incubations. Culture samples were collected from each of the mixed cultures at 0, 6 and 24 h of incubation for colorimetric determination of ammonia and bacteriological enumeration of *Campylobacter* on Campy-Cefex agar. Portions of fluid samples from the respective porcine or bovine mixed culture incubations were analysed for concentrations of volatile fatty acid by gas chromatography (Lambert & Moss, 1972; Salanitro & Muirhead, 1975) and fluid samples from mixed cultures of bovine microbes were also subjected to GC analysis for determination of free thymol. Measurements of pH were made, using remaining volumes and a pH meter.

2.4. Statistics

All incubations were performed in triplicate. Concentrations of ammonia, volatile fatty acids and $\log_{10}$ transformations of cfu/ml of *C. coli* and *C. jejuni* were tested for treatment effects at each sample time by a general analysis of variance. Treatment means were compared to controls, using a two-sided Dunnett's multiple comparison procedure. Differences were considered significant at $p \leqslant 0.05$. All analyses were performed using Statistix© 9 Analytical Software (Tallahassee, FL, USA).

3. Results

3.1. Pure and co-culture studies with P. distasonis

Viable cell counts of *C. coli* during pure culture in medium supplemented with 1 mM thymol were lower than those of

non-thymol-treated control cultures after 24 h of incubation ($p < 0.05$) but only tended ($p = 0.0537$) to be lower after 48 h (Fig. 1A). When grown in pure culture with 1 mM added mM thymol-β-D-glucopyranoside, viable counts of *C. coli* were greater by nearly 2.0 $\log_{10}$ cfu/ml ($p < 0.05$) than those of controls after a 24 h culture but were lower, albeit not significantly, by 1.88 $\log_{10}$ cfu/ml than those of controls after 48 h of culture. During co-culture with *P. distasonis*, viable counts of *C. coli* supplemented with 1 mM thymol were 3.54 and 4.30 $\log_{10}$ cfu/ml lower ($p < 0.05$) than those of controls after 24 and 48 h of culture, respectively (Fig. 1B). Viable *C. coli* counts did not differ from control counts after 24 h of co-culture in medium supplemented with 1 mM added thymol-β-D-glucopyranoside but were 4.45 $\log_{10}$ cfu/ml lower ($p < 0.05$) than control counts after 48 h of culture with 1 mM thymol-β-D-glucopyranoside (Fig. 1B).

For pure cultures of *C. jejuni*, counts were 1.88 and 4.80 $\log_{10}$ cfu/ml lower ($p < 0.05$) than those of controls after 24 and 48 h of incubation in Bolton broth supplemented with 1 mM thymol but were not lower in cultures treated with 1 mM thymol-β-D-glucopyranoside (Fig. 2A). Conversely, when co-cultured with a β-glycoside-hydrolysing *P. distasonis*, counts of *C. jejuni* were 6.43 and 6.87 $\log_{10}$ cfu/ml lower ($p < 0.05$) than those of controls after 24 and 48 h, respectively, in cultures treated with 1 mM thymol and were 3.72 $\log_{10}$ cfu/ml lower ($p < 0.05$) after 48 h in cultures treated with 1 mM thymol-β-D-glucopyranoside (Fig. 2B). As observed with *C. coli*, viable cell counts of *C. jejuni* were not significantly lower after 24 h of co-culture with *P. distasonis* in Bolton broth supplemented with 1 mM thymol-β-D-glucopyranoside, thus indicating that there was likely a lag in the hydrolysis of the β-glycoside and a subsequent delay in the establishment of inhibitory concentrations of free thymol.

Rates of ammonia accumulation (mean ± SD) in 1 mM thymol-treated pure cultures of *C. coli* (0.01 ± 0.01 μmol/ml per h) were reduced ($p < 0.05$) when compared to controls (0.08 ± 0.01 μmol/ml per h) but were not reduced in pure cultures treated with 1 mM thymol-β-D-glucopyranoside (0.09 ± 0.01 μmol/ml per h). However, significant differences in rates of ammonia accumulation were not observed during co-culture of *C. coli* with *P. distasonis* (0.10 ± 0.05, 0.04 ± 0.07 and 0.04 ± 0.01 μmol/ml per h for control, 1 mM thymol- and 1 mM thymol-β-D-glucopyranoside-treated

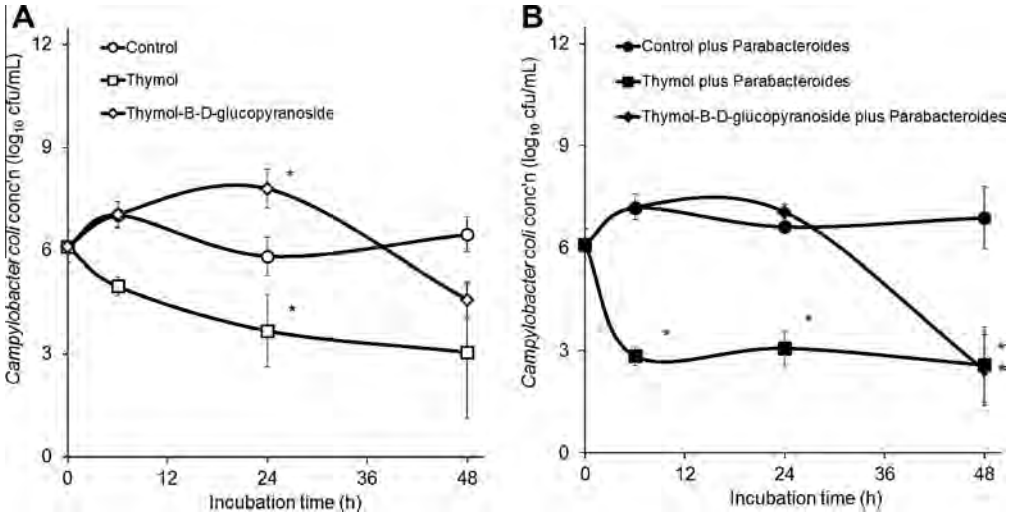

Fig. 1. Viable cell counts during incubation of *Campylobacter coli* in pure culture (A) or co-culture with *Parabacteroides distasonis* (B) incubated anaerobically (N_2) at 39 °C in Bolton broth supplemented without (circles) or with either 1 mM thymol (squares) or 1 mM thymol-β-D-glucopyranoside (diamonds). Values are the means ± SD from $n = 3$ cultures and asterisks indicate values differing from control values ($p < 0.05$).

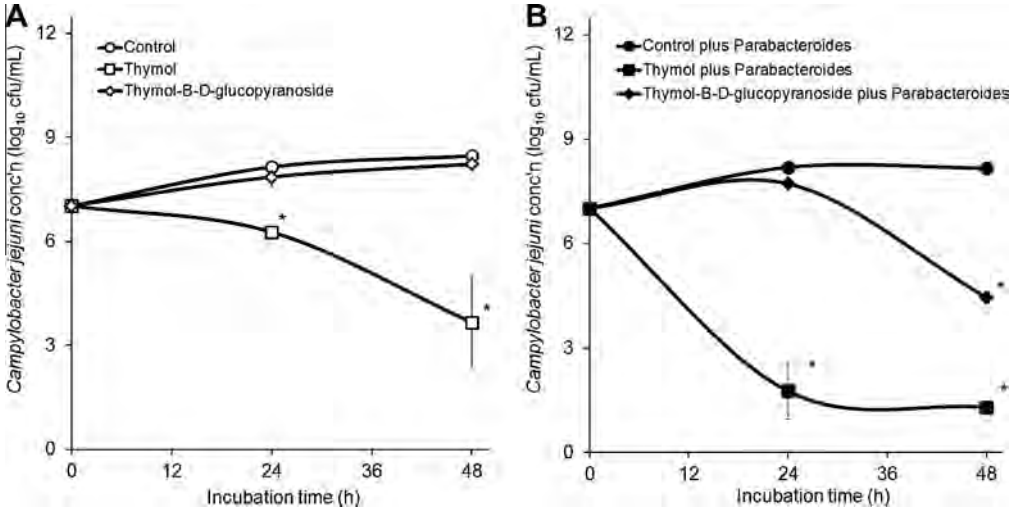

Fig. 2. Viable cell counts during incubation of *Campylobacter jejuni* in pure culture (A) or co-culture with *Parabacteroides distasonis* (B) incubated anaerobically (N_2) at 39 °C in Bolton broth supplemented without (circles) or with either 1 mM thymol (squares) or 1 mM thymol-β-D-glucopyranoside (diamonds). Values are the means ± SD from $n = 3$ cultures and asterisks indicate values differing from control values ($p < 0.05$).

cultures, respectively). In the case of *C. jejuni*, rates of ammonia accumulation were likewise reduced ($p < 0.05$) from those of controls by pure cultures treated with 1 mM thymol (0.13 ± 0.01 and 0.07 ± 0.01 μmol/ml per h for control and thymol-treated cultures, respectively) but were not reduced in pure cultures treated with 1 mM thymol-β-D-glucopyranoside (0.12 ± 0.01 μmol/ml per h). Rates of ammonia accumulation during co-culture of *C. jejuni* with *P. distasonis* were reduced ($p < 0.05$) from those of controls (0.22 ± 0.07 μmol/ml per h) by 1 mM thymol (0.03 ± 0.01 μmol/ml per h) but not 1 mM thymol-β-D-glucopyranoside (0.28 ± 0.02 μmol/ml per h).

In thymol-treated cultures, thymol concentrations measured in fluid samples collected after 6, 24 and 48 h incubations of pure cultures of *C. jejuni* averaged (±SD) 0.52 ± 0.02, 0.48 ± 0.05 and 0.44 ± 0.01 μmol/ml and did not differ from each other ($p > 0.05$). However, these concentrations were decreased ($p < 0.05$) by approximately 48–56% from the initial 1 mM thymol. Similarly, thymol concentrations averaged 0.58 ± 0.06, 0.64 ± 0.08 and 0.50 ± 0.04 μmol/ml in fluid samples collected after 6, 24 and 48 h incubations of co-cultures of *C. jejuni* and *P. distasonis* and were decreased ($p < 0.05$) by 36–50% from the initial 1 mM added thymol concentration. In co-cultures treated with 1 mM thymol-β-D-glucopyranoside, thymol accumulations were much lower, even by 48 h of incubation (0.37 ± 0.02 μmol/ml) and never exceeded 0.2 μmol/ml in pure cultures treated with 1 mM thymol-β-D-glucopyranoside, the latter possibly added as a potential impurity, albeit at low concentration, within the stock thymol-β-D-glucopyranoside solution.

In the case of *C. jejuni*, pH measurements at the end of the 48 h incubation were unaffected by treatment during pure culture ($p = 0.1679$) and averaged 7.40 ± 0.19. An effect of treatment was observed ($p = 0.0009$) on pH during co-culture, however, with the pH measured after 48 h of co-culture with thymol (7.47 ± 0.03) being higher ($P < 0.05$) than that measured in controls (7.22 ± 0.02). The pH in co-cultures treated with thymol-β-D-glucopyranoside (7.23 ± 0.07) did not differ from that of controls. Measurements of pH were not made during pure or co-culture of *C. coli*.

3.2. Mixed culture studies with porcine and bovine faecal populations

When grown in mixed culture with freshly collected populations of porcine faecal microbes, major effects of thymol or thymol-β-D-glucopyranoside treatments on recovery of *C. coli* were observed (Fig. 3A). For the mixed porcine populations treated with 1 mM thymol, viable counts of *C. coli* were 3.11 and 3.26 $\log_{10}$ cfu/ml lower ($p < 0.05$) than those of untreated controls after 6 and 24 h incubations, respectively (Fig. 3A). For the mixed porcine populations treated with 1 mM thymol-β-D-glucopyranoside, *C. coli* counts were 2.03 and 3.26 $\log_{10}$ cfu/ml lower ($p < 0.05$) than those of untreated controls after 6 and 24 h incubations, respectively (Fig. 3A). In the case of *C. jejuni* incubated with the mixed populations of bovine faecal microbes, a major effect of thymol treatment was observed, with *C. jejuni* counts being 3.73 and 3.06 $\log_{10}$ cfu/ml lower ($p < 0.05$) than those of untreated controls after 6 and 24 h incubations, respectively (Fig. 3B). In the mixed bovine cultures treated with 1 mM thymol-β-D-glucopyranoside, *C. jejuni* counts did not differ ($p > 0.05$) from the untreated control counts at the 6 h sampling time but were 2.96 $\log_{10}$ cfu/ml lower ($p < 0.05$) than the untreated controls after 24 h of incubation (Fig. 3B). Not unexpectedly, appreciable differences between untreated and ethanol-treated (0.6% vol/vol) controls were not observed and thus results from the latter are not presented.

Rates of ammonia accumulation were reduced ($p < 0.05$) from those of untreated controls (0.26 ± 0.14 and 0.70 ± 0.01 μmol/ml per h for porcine and bovine faecal microbes, respectively) during mixed culture, with rates being 0.01 ± 0.01 and 0.04 ± 0.04 μmol/ml per h in porcine cultures and being 0.56 ± 0.03 and 0.17 ± 0.04 μmol/ml per h in bovine cultures treated with 1 mM thymol or thymol-β-D-glucopyranoside, respectively.

A major effect of treatment was observed on production of the fermentation acids, acetate and propionate, in the mixed cultures of porcine (Fig. 4A and B) faecal microbes incubated 24 h, with production by cultures treated with 1 mM thymol-β-D-glucopyranoside being in lower amounts than ($p < 0.05$) those of the controls. Effects of treatment on accumulations of butyrate, valerate and branched chain fatty acids, isobutyrate and isovalerate, were also observed, with concentrations being decreased ($p < 0.05$) from those of controls in the mixed cultures of porcine faecal microbes supplemented with 1 mM thymol-β-D-glucopyranoside (Fig. 4A and B). Accumulations of the branch chain volatile fatty acids, isobutyrate and isovalerate, by mixed porcine cultures treated with 1 mM thymol were lower ($p < 0.05$) than those of controls but accumulations of the other volatile fatty acids did not differ. A major effect of treatment was observed on accumulations of acetate and propionate during mixed culture of *C. jejuni* with bovine faecal microbes (Fig. 5A and B), with accumulations in cultures

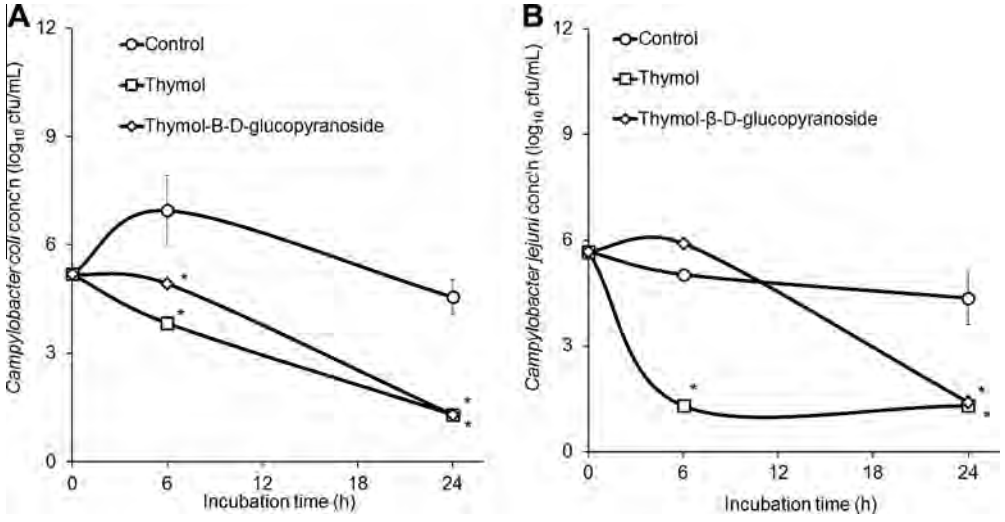

Fig. 3. Viable cell counts of *Campylobacter coli* during mixed culture of porcine faecal microbes (A) or *Campylobacter jejuni* during mixed culture of bovine faecal microbes (B) incubated anaerobically (N_2) at 39 °C in Bolton broth supplemented without (circles) or with either 1 mM thymol (squares) or 1 mM thymol-β-D-glucopyranoside (diamonds). Values are the means ± SD from $n = 3$ cultures and asterisks indicate values differing from control values ($p < 0.05$).

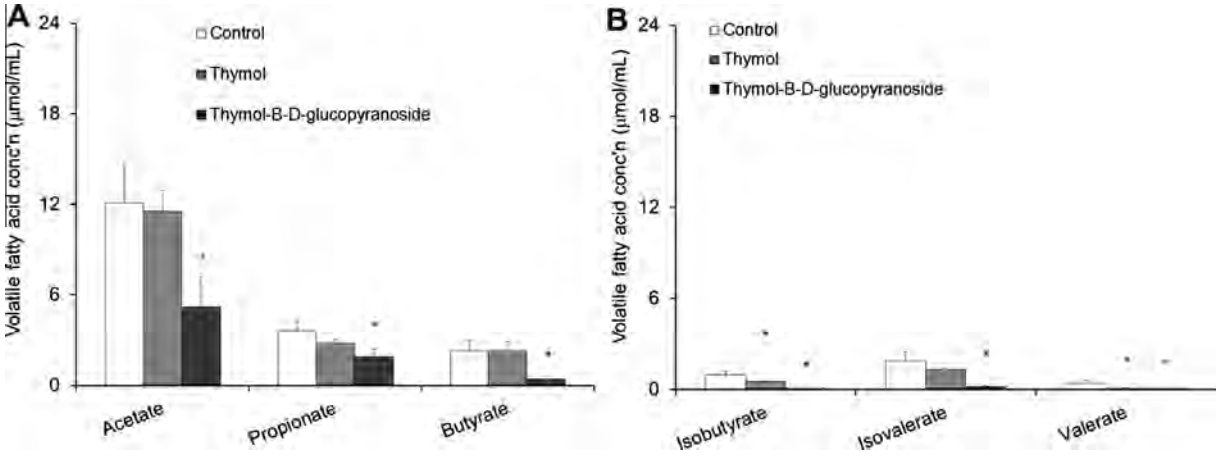

Fig. 4. Production of fermentation acids during mixed culture of *Campylobacter coli* with porcine gut microbes. Cultures were incubated anaerobically (N_2) at 39 °C in Bolton broth supplemented without (open bars) or with either 1 mM thymol (shaded bars) or 1 mM thymol-β-D-glucopyranoside (dark bars). Values are the means ± SD from $n = 3$ cultures and asterisks indicate values differing from control values ($p < 0.05$).

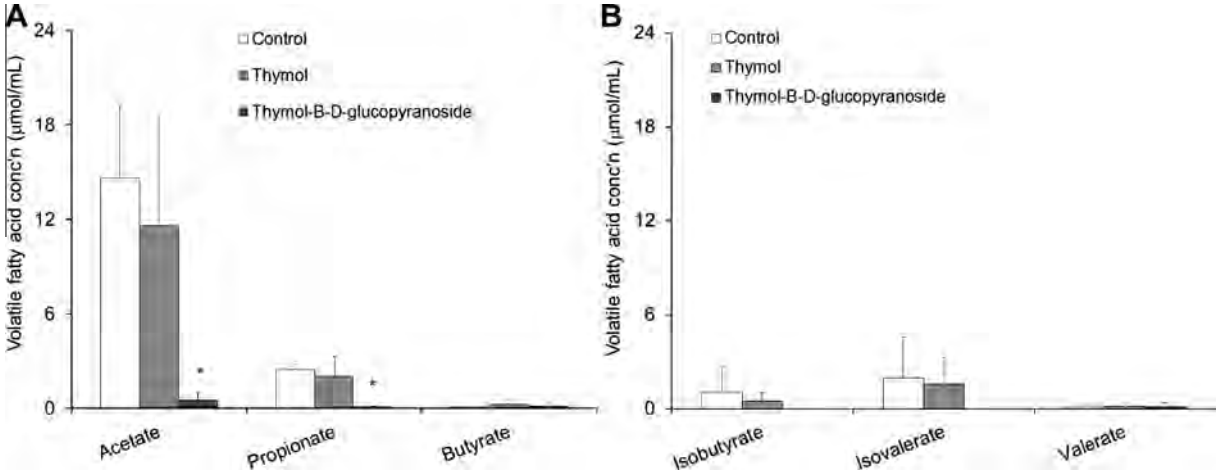

Fig. 5. Accumulation of fermentation acids during mixed culture of *Campylobacter jejuni* with bovine gut microbes. Cultures were incubated anaerobically (N_2) at 39 °C in Bolton broth supplemented without (open bars) or with either 1 mM thymol (shaded bars) or 1 mM thymol-β-D-glucopyranoside (dark bars). Values are the means ± SD from $n = 3$ cultures and asterisks indicate values differing from control values ($p < 0.05$).

treated with 1 mM thymol-β-D-glucopyranoside being lower than those of controls. Significant treatment effects were not observed ($p > 0.05$) on accumulations of any of the other volatile fatty acids in the mixed cultures of bovine faecal microbes. A major effect of treatment was observed on final pH during mixed culture of the porcine and bovine faecal microbes. For the mixed porcine cultures, the final pH was higher ($p < 0.05$) in controls (6.90 ± 0.03) than in cultures treated with 1 mM thymol or 1 mM thymol-β-D-glucopyranoside (6.75 ± 0.02 and 6.59 ± 0.09, respectively). In the case of the mixed bovine cultures, the final pH was higher ($p < 0.05$) in the thymol-treated (7.34 ± 0.31) cultures than in controls (6.62 + 0.07) but the pH in cultures treated with thymol-β-D-glucopyranoside (6.67 ± 0.11) did not differ from those of the controls. Free thymol accumulations in fluid samples collected from mixed cultures of bovine faecal microbes incubated with the addition of 1 mM free thymol were 0.93 ± 0.36 and 0.77 ± 0.21 μmol/ml at 6 and 24 h and were not different ($p > 0.05$) from the amount added. Free thymol accumulations in fluid samples collected from mixed bovine faecal cultures treated with 1 mM added thymol-β-D-glucopyranoside were 0.14 ± 0.02 and 1.28 ± 0.49 μmol/ml at 6 and 24 h.

4. Discussion

Results from the present study support results from earlier work of Anderson et al. (2009) showing that 1 mM thymol markedly reduced the survivability of *C. coli* and *C. jejuni*, in both pure and mixed culture with porcine and bovine gut microbes. Thymol is bactericidal to important foodborne pathogens with minimum bactericidal concentrations reported to be 0.66, 1.10 and 1.55 mM for *Escherichia coli* K88, *E. coli* O157:H7 and *Salmonella enterica* serovar Typhimurium DT104, respectively (Si et al., 2006). Total anaerobes from the pig intestine were less susceptible to 1.72 mM thymol (Michiels, Missotten, Fremaut, De Smet, & Dierick, 2007). Mechanistically, thymol is thought to exert its bactericidal effect via disruption of the bacterial cell wall (Burt, 2004), although the ability of thymol to inhibit deaminase activity has been proposed as a potential mechanism limiting the growth of assacharolytic *Campylobacter* (Anderson et al., 2009). With respect to the latter mechanism, rates of ammonia accumulations were decreased in the present study by 1 mM thymol during pure culture of *C. coli* and *C. jejuni*, as well as during co-culture with *P. distasonis*. Rates of ammonia accumulation during co-culture of

C. coli were not decreased significantly, likely because of the high amount of variability in ammonia measurements in these cultures.

Despite the clearly evident bactericidal activity of thymol, its use as a preharvest feed additive to reduce the colonization of foodborne pathogens in the lower gut is likely to be limited because it is rapidly and extensively absorbed or degraded in the proximal alimentary tract (Anderson et al., 2012; Michiels et al., 2010). Consequently, researchers have recognised a need for encapsulation or other protection technologies to deliver effective concentrations of thymol to the lower gut to achieve *in vivo* reductions of *Campylobacter* (Anderson et al., 2012; Michiels et al., 2010). Whereas application of natural and synthetic glycosidic conjugates to deliver pharmaceuticals to the colon has been used previously for a variety of other compounds (Chourasia & Jain, 2003), the use of β-glycosides to deliver thymol to the lower gut has only recently been investigated. Conceptually, if constructed properly, intact β-glycosides should be resistant to absorption. In that regard, recent evidence has indeed shown that intact thymol-β-D-glucopyranoside is much more resistant to absorption, being absorbed across everted porcine jejunal segments at 1/3 the rate of free thymol (Petrujkić et al., 2013). Additionally, in monogastric animals, the intact glycosides should be resistant to hydrolysis because higher animals are limited in their ability to produce β-glycoside-hydrolysing enzymes in the proximal alimentary tract. On the other hand, β-glycoside-hydrolysing activity is expressed by populations of gut microbes colonizing the lower intestinal tract. For example, in pigs, β-glycosidase activity is low or absent in the stomach and proximal small intestine and does not become appreciable until the distal small intestine, caecum or large intestine, where populations of competent hydrolysing bacteria are established (Friend, 1992). β-Glycosidase-expressing bacteria include certain species belonging to Bacteroides, Bifidobacteria, Clostridia, Enterobacteria, Enterococci and Lactobacilli (Hawksworth, Drasar, & Hill, 1971). Evidence from the present co-culture studies indicates that the *P. distasonis* used here possesses β-glycosidase activity, which is consistent with characteristics ascribed to the genus and species of this gut bacterium (Sakamoto & Benno, 2006). In the present study, the 1 mM thymol-β-D-glucopyranoside treatment was inhibitory to *C. coli* and *C. jejuni*, but this activity was significant after 48 h only, when co-cultured with *P. distasonis*, or when cultured with mixed populations of gut bacteria. These results provide evidence that the bactericidal activity of thymol-β-D-glucopyranoside was dependent on the presence of microbial populations possessing enzymatic activity capable of hydrolysing the glycosidic bond. Conversely, the lack of an effect of thymol-β-D-glucopyranoside on the recovery of viable *C. coli* and *C. jejuni* during pure culture is not unexpected, considering that there is no evidence that these two *Campylobacter* species express β-glycosidase (Columina, Villar, Buesa, & Borras, 1997). Furthermore, only very low amounts of free thymol (<0.20 mM) were measured in pure cultures of *C. jejuni* treated with thymol-β-D-glucopyranoside and this may likely have been added as a contaminant of the synthesized thymol-β-D-glucopyranoside. Similarly, ammonia accumulations were not significantly affected by thymol-β-D-glucopyranoside during pure culture of *C. coli* and *C. jejuni*, which suggests that appreciable amounts of free thymol were not made available to inhibit amino acid deamination. Significant effects of thymol-β-D-glucopyranoside were not observed on ammonia accumulations during co-culture and this is contrary to what would be expected since the β-glycoside-hydrolysing activity of *P. distasonis* should liberate free thymol. It is possible, though, that the considerable contribution of *P. distasonis* to ammonia production may have masked an effect of any thymol potentially liberated during co-culture. Moreover, the bactericidal effect of the 1 mM thymol-β-D-glucopyranoside against *C. coli* and *C. jejuni* during co-culture was not apparent until the 48 h sampling period, which suggests that the liberation of free thymol from the glucose conjugate occurred slowly and late during culture. The low recovery of free thymol (0.37 ± 0.02 mM) from incubation fluids collected after 48 h co-cultures of *C. jejuni* and *P. distasonis* provide further evidence that the hydrolysis occurred slowly. Effects of thymol and thymol-β-D-glucopyranoside treatment on ammonia production during mixed cultures were dependent on the source of the mixed microbial population. For instance, significant effects of 1 mM thymol or 1 mM thymol-β-D-glucopyranoside treatment on ammonia accumulations were observed during culture of *C. coli* and *C. jejuni* with the respective porcine and bovine faecal populations, but again this inhibition did not occur until late during culture. When measured in fluid samples collected from the mixed bovine cultures, appreciable free thymol did not accumulate in the cultures treated with 1 mM thymol-β-D-glucopyranoside until after 24 h.

From a practical perspective, it is reasonable to suspect that thymol-β-D-glucopyranoside would be most suited for use as a feed additive in pigs because little hydrolysis of the glucose conjugate would be expected to occur within the pig stomach or proximal small intestine. Conversely, a major challenge to the use of thymol-β-D-glucopyranoside or other β-glycoside-linked conjugates in ruminants or poultry is the presence of competent β-glycoside-hydrolysing microbial populations in their rumen or crop. In a recent live animal study, oral administration of thymol-β-D-glucopyranoside reduced (by more than 10-fold) the recovery of *Campylobacter* from the crop, but not the caeca, of market-aged broilers when compared to placebo- or free thymol-treated broilers (Epps et al., 2015). Thus, orally administered thymol-β-D-glucopyranoside, but not free thymol (each administered to achieve approximately 1 mM in the total avian gastrointestinal tract), was retained and able to be hydrolysed to biologically active free thymol in the crop but was not sufficiently protected to allow passage of efficacious amounts of the intact glycoside to the caeca. It is possible that higher doses of thymol-β-D-glucopyranoside may allow sufficient amounts to escape hydrolysis in the crop or rumen and subsequent absorption, but this has yet to be tested. Alternatively, it is possible that additional encapsulation or other protective technologies could be used to protect passage of the intact glycoside through pre-gastric fermentation sites, thereby promoting its delivery to the lower gut.

In the present study, volatile fatty acid production by mixed cultures of porcine or bovine faecal bacteria was unaffected by 1 mM free thymol but this was not unexpected, as earlier work had shown that appreciable inhibition of volatile fatty acid production by mixed populations of rumen microbes was not achieved until thymol doses exceeded 1–3 mM (Castillejos, Calsamiglia, & Ferret, 2006; Macheboeuf, Morgavi, Papon, Mouset, & Arturo-Schaan, 2008). Importantly, however, results from the present incubations with mixed populations of bovine faecal microbes revealed that thymol-β-D-glucopyranoside treatment markedly inhibited accumulations of major fermentative acids, acetate and propionate, possibly because of enhanced active or permease-mediated uptake of the glycoside by the microbial population. This finding indicates that the β-glycoside was a potent inhibitor of fermentation which will likely limit the amount that can be fed at one time and potentially restrict its use as a terminal preharvest feed additive to be administered in the animals' last meal or perhaps in the water upon arrival at the processing plant.

In conclusion, results from the present studies extend the findings of thymol's antimicrobial activity in addition to addressing the issues pertaining to absorption within the stomach and small intestine. These results demonstrate that thymol and thymol-β-D-glucopyranoside inhibited *C. coli* and *C. jejuni* during pure culture, of mixed populations of bovine, porcine gut microbes, although the bactericidal activity of thymol-β-D-glucopyranoside

was dependent on populations contributing β-glycoside-hydrolysing activity. Further studies are clearly needed to elucidate practical and economic issues pertaining to its use as a feed additive.

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